Cooper, M. D., Chase, H. P., Lowman, J. T., Krivit, W., & Good, R. A. (1968) *Am. J. Med. 44*, 499–513.
Foley, G. E., Lazarus, H., Farber, S., Uzman, B. G., Boone, B. A., & McCarthy, R. E. (1965) *Cancer (Philadelphia) 18*, 522–529.
Griffiths, G., Quinn, P., & Warren, G. (1983) *J. Cell Biol. 96*, 835–850.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Minowada, J., Ohnuma, T., & Moore, G. E. (1972) *J. Natl. Cancer Inst. (U.S.) 49*, 891–895.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Parkman, R., Remold-O'Donnell, E., Kenney, D. M., Perrine, S., & Rosen, F. S. (1981) *Lancet ii*, 1387–1389.
Remold-O'Donnell, E. (1985) *J. Exp. Med. 162*, 2142–2155.
Remold-O'Donnell, E., Kenney, D. M., Parkman, R., Cairns, L., Savage, B., & Rosen, F. S. (1984) *J. Exp. Med. 159*, 1705–1723.
Remold-O'Donnell, E., Davis, A. E., III, Kenney, D. M., Bhaskar, K. R., & Rosen, F. S. (1986) *J. Biol. Chem. 261*, 7526–7530.
Remold-O'Donnell, E., Zimmerman, C., Kenney, D., & Rosen, F. S. (1987) *Blood* (in press).
Robbins, P. W., Hubbard, S. C., Turco, S. J., & Wirth, D. F. (1977) *Cell (Cambridge, Mass.) 12*, 893–900.
Roberts, B. E., & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2330–2334.
Roth, J. (1984) *J. Cell Biol. 98*, 399–406.
Tarentino, A. L., Plummer, T. H., Jr., & Maley, F. (1974) *J. Biol. Chem. 249*, 818–824.
Tarentino, A. L., Gomez, C. M., & Plummer, T. H., Jr. (1985) *Biochemistry 24*, 4665–4671.
Tkacz, J. S., & Lampen, J. O. (1975) *Biochem. Biophys. Res. Commun. 65*, 248–257.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wiskott, A. (1937) *Monatsschr. Kinderheilkd. 68*, 212–216.

Continuous Monitoring of Adenosine 5′-Triphosphate in the Microenvironment of Immobilized Enzymes by Firefly Luciferase†

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ABSTRACT: The study of enzymes sequestered in artificial or biological systems is generally conducted by indirect methodology with macroscopic measurements of reactants in the bulk medium. This paper describes a new approach with firefly luciferase to monitor ATP concentration directly in the microenvironment of enzymes producing or consuming ATP. Upon addition of ATP to immobilized firefly luciferase, the onset of light production is slower than that observed with the soluble enzyme, due to a slower diffusion of ATP to the immobilized enzyme. With immobilized pyruvate kinase, a relative accumulation of ATP inside the beads is demonstrated, as measured with coimmobilized firefly luciferase. The accumulation of product (ATP) is enhanced when the bead suspension is not stirred. This ATP in the beads is relatively inaccessible to soluble hexokinase added to the bulk medium. Similarly, a rapid ATP depletion in the microenvironment of immobilized hexokinase is demonstrated. This microscopic event is kinetically distinguishable from the slower macroscopic depletion of substrate in the bulk medium. The rate of depletion in the microenvironment depends on the local activity of the immobilized enzyme but not on the total amount of enzyme in suspension, as does the macroscopic phenomenon. The theoretical principles for the interaction of diffusion and catalysis in these systems are briefly summarized and discussed. These results are relevant to various molecular mechanisms proposed for membrane-bound enzyme action and regulation, derived from macroscopic kinetic measurements assuming a negligible diffusion control.

Enzymes and enzymic systems are frequently associated with membranous structures of definite topology (De Pierre & Ernster, 1977) and thus are associated with, or segregated into, different compartments within the cell. Consequently, in addition to enzymic steps, metabolic processes in situ include physical phenomena such as transport, partition, or diffusion of metabolites between the *loci* of the catalytic reactions (Katchalski et al., 1971; Engasser & Horvath, 1976; Goldstein, 1976; Laidler & Bunting, 1980). Unfortunately, due to great experimental difficulties, little is known at present about diffusional resistances inside the cell. Most metabolic studies are done through macroscopic measurements of the concentration of reactants in the bulk medium surrounding solubilized or particulate systems. These measurements reflect both local catalytic events and the transfer of reactants and intermediates between the individual catalytic units or complexes. The results of such studies are often interpreted in terms of steady-state enzyme kinetics, implicitly assuming a large excess of reactants over the enzymes and a homogeneous distribution of freely diffusible intermediates and products. This situation does not apply generally to cellular metabolism, and the physical phenomena are likely to be of physiological importance in view of the intricate structure of the cell and the large concentration of enzymes in vivo (Ottaway & Mowbray, 1977; Weber & Bernhard, 1982; Srere, 1984).

Immobilization of enzymes on solid supports allows the design of artificial, relatively simple and defined systems of

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concentrated proteins to study such problems. It has been shown both theoretically and experimentally that the interaction of reactants with the support determines their distribution within the matrix and in the bulk medium during catalysis (Katchalski et al., 1971; Engasser & Horvath, 1976; Goldstein, 1976; Laidler & Bunting, 1980). As a rule, diffusional restrictions tend to reduce the apparent efficiency of exogeneous substrate utilization by immobilized enzymes. This is due to a local depletion of substrate and accumulation of product in the environment of the enzyme at steady state. On the other hand, in coimmobilized multienzyme systems, the efficiency of the overall sequence of coupled reactions increases due to the proximity of consecutively acting enzymes in the microenvironment (Katchalski et al., 1971; Ottaway & Mowbray, 1977; Mosbach, 1978; Weber & Bernhard, 1982; Srere, 1984).

In previous studies, the heterogeneous distribution of reactants in such systems has been assessed indirectly from macroscopic measurements (in the bulk medium) only. However, it is possible to detect directly reactants in the microenvironment of immobilized enzymes with coimmobilized coupled enzymes or chemical probes. We reported recently the possible application of immobilized firefly luciferase on Sepharose beads to monitor ATP produced by coimmobilized pyruvate kinase in the bead, by following the luminescence evolving in the presence of luciferin (DeLuca, 1984).

FL[1] catalyzes the oxidative decarboxylation of D-luciferin, following the activation of luciferin with ATP. During the course of the reaction, a photon is produced with a quantum yield close to 1. Under proper experimental conditions, the light output is proportional to the concentration of ATP (DeLuca & McElroy, 1978; Schram et al., 1981; Lemasters & Hackenbrock, 1976). This feature permits quantitation of [ATP] in the direct environment of the enzyme. Moreover, since photons are not subject to physical (e.g., diffusional) restrictions as are chemical products, the system has obvious advantages over other localized probes.

We describe here the use of immobilized FL as a localized probe for ATP, and outline the basic principles for quantitative analysis. Coimmobilized or separately immobilized FL coupled to other immobilized kinases monitors ATP concentration in the microenvironment or the bulk medium, respectively. The results demonstrate the relative accumulation and depletion of ATP in the microenvironment of coimmobilized PK and HK, respectively. Such phenomena are not detected in the bulk medium. This method enables the assessment of concentration gradients of ATP between both compartments. To our knowledge, this is the first time such gradients of reactants have been directly demonstrated.

Materials and Methods

Materials. Yeast hexokinase (EC 2.7.1.1) and rabbit muscle pyruvate kinase (EC 2.7.1.40) were purchased from Calbiochem and Boehringer Mannheim, respectively. Firefly luciferase was isolated from *Photinus pyralis* lanterns and purified as described (DeLuca & McElroy, 1978). Sepharose 4B beads were from Pharmacia, and cyanogen bromide was from Eastman-Kodak. Firefly luciferin was synthesized and purified according to Bowie (1978). All other reagents were of analytical grade.

[1] Abbreviations: [X], concentration of X; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FL, firefly luciferase; $h\nu$, photon; HK, hexokinase; LH_2, firefly D-luciferin; P, oxyluciferin; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PP_i, inorganic pyrophosphate; tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Table I: Summary of Data on Immobilized Enzyme Preparations[a]

preparation	specific activities[b] (% yield)		
	FL (V/g)	PK (IU/g)	HK (IU/g)
(I) Im·FL)	1300 (76)	0.0	0.0
(II) Im·PK	0	7.5 (90)	0.0
(III) Im·HK	0	0.0	11.5 (39)
(IV Im·FL:PK	1500 (88)	7.0 (83)	0.0
(V) Im·FL:HK	1200 (71)	0.0	11.2 (36)

[a] Immobilization of enzymes on CNBr-activated Sepharose 4B was done as described under Materials and Methods, and so the measurements of activities. The numbers of parentheses represent the yield of immobilization as percentage of the initial activities measured in aliquots of the enzyme solutions previous to the procedure. The activities are expressed in international units (PK and HK) or volts (FL) per gram of Sepharose. [b] The values represent minimal activities due to their possible underestimation because of diffusional restrictions.

Methods. The enzymes were immobilized on Sepharose 4B beads by the CNBr procedure (Axen et al., 1967) in the presence of a saturating concentration (5 mg/mL) of BSA as previously described (Wiehnhausen et al., 1982), so that the microenvironment of the bound enzymes represents a "buffered" milieu as in most cellular compartments. The beads were extensively washed and finally resuspended at 10% (v/v) in a storage medium consisting of 50 mM potassium tricine, pH 8.0, 10% glycerol, 0.5 mM DTT, and 0.02% NaN_3.

The standard reaction mix contained 50 mM potassium tricine, pH 8.0, 2 mM K-EDTA, 10 mM $MgCl_2$, and 0.5 mg/mL BSA in a final volume of 2.5 or 1.0 mL for spectrophotometric or luminometric determinations, respectively.

PK and HK activities were determined by coupling the reactions to lactate dehydrogenase/NADH and glucose-6-phosphate dehydrogenase/NAD^+ in excess (×100), respectively, and following the change in absorbance at 340 nm (Worthington Manual, 1978). The measurements were performed in a well-stirred cuvette at room temperature with a Cary 219 spectrophotometer.

FL activity was determined as the peak light intensity produced upon the rapid addition of a saturating concentration of ATP (4 mM) to a reaction mix containing the enzyme and 0.1 mM LH_2, on an LKB 1250 luminometer. A description of the immobilized enzyme preparations used in this work is given in Table I.

Continuous light emission was monitored under similar conditions with much lower ATP concentrations. Reactions with immobilized enzymes were followed at room temperature, and the suspensions were stirred in the luminometer chamber by a mixing device developed in this laboratory. Additions were made in small volumes (10 μL) with a PM America 109N rapid-injection device.

Calculations of rate constants for pseudo-first-order processes were done from the linear portion (15–90% completion) of semilogarithmic plots of the relative variation of light intensity with time (Neet & Ainslie, 1980).

Results and Discussion

Studies on Immobilized Firefly Luciferase

FL catalyzes the following sequence of reactions (DeLuca & McElroy, 1978):

$$FL + LH_2 + ATP \overset{Mg^{2+}}{\leftrightarrow} FL{\cdot}LH_2{\cdot}ATP \text{ (rapid equilibrium)} \quad (1)$$

$$FL{\cdot}LH_2{\cdot}ATP \rightarrow FL{\cdot}LH_2{-}AMP + PP_i \quad (2)$$

$$FL{\cdot}LH_2{-}AMP + O_2 \rightarrow FL{\cdot}P^* + AMP + CO_2 \quad (3)$$

$$FL{\cdot}P^* \rightarrow FL{\cdot}P + h\nu \text{ (fast)} \quad (4)$$

$$FL{\cdot}P \rightarrow FL + P \quad (5)$$

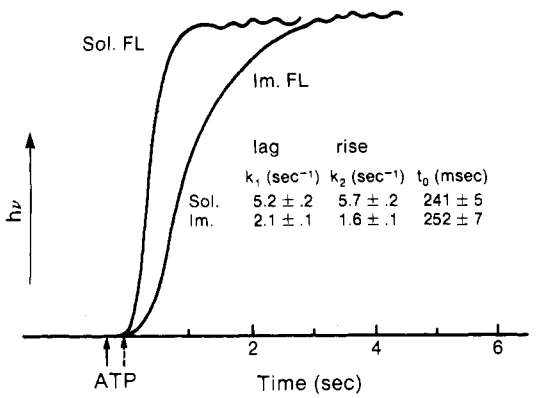

FIGURE 1: Light evolution from soluble and immobilized FL. The reaction conditions were as described under Materials and Methods. The reaction mix contained 5 nM soluble FL or 0.5 mg of Sepharose/mL of immobilized FL from preparation I (see Table I). The final ATP concentration was 100 nM. The light outputs were normalized. (Inset) Calculated kinetic parameters for the biphasic rise of luminescence (see text). The data were fitted to eq A2, in which the variable t was replaced by $t - t_0$; where t_3 represents the time elapsed between the actuation of ATP injection (full arrow) and the true mixing time (dashed arrow).

Steps 2 and 5 are reversible, but the backward reaction may be neglected at the early stage of catalysis. In all the experiments described below, FL was preequilibrated with a saturating concentration of LH_2, and light production was initiated by addition of ATP.

When all substrates are in excess, steps 1–4 proceed relatively rapidly and the reaction is severely limited at steady state by the product release step 5 (Schram et al., 1981; Lemasters & Hackenbrock, 1976). When ATP is added to FL and other substrates, after a short lag a rapid rise in light intensity occurs, followed by a slower decay representing the accumulation of an inactive FL·P complex (not shown) to a steady-state value (DeLuca & McElroy, 1978).

However, upon reducing the velocity of step 1 by use of very low [ATP], after a biphasic rise no fast decay in light intensity is observed (Figure 1). This indicates that step 5 is no longer limiting the steady-state rate of light production (DeLuca & McElroy, 1978; Lemasters & Hackenbrock, 1976).

The pre-steady-state kinetics of light production at low [ATP] may be treated as a sequence of two first-order (limiting) processes (steps 2 and 3), keeping in mind that light intensity represents the rate of photon production (see Appendix A). The first process, during which bound precursors accumulate (step 2) is represented by a lag in light production after the rapid addition of ATP. The second process represents the formation of an intermediate at the excited state (step 3), whose rapid decay to the ground state results in photon production (step 4). The rate constants of these processes are independent of [FL] and [ATP] (Lemasters & Hackenbrock, 1976). When the reaction is monitored for longer times, an exponential decay is observed, until all the ATP is consumed. Addition of another aliquot of ATP restores about 95% of the original light output (not shown). From these experiments, a turnover rate of about 0.3 mol of ATP·(mol of FL)$^{-1}$·min^{-1} may be calculated, under the conditions used in Figure 1.

An important consideration is that FL, because of its slow turnover, does not use significant amounts of ATP, compared with other enzymes in similar conditions. Moreover, at low [ATP], virtually no product inhibition occurs. Thus, we expect that the light output at steady state should remain proportional to the total amount of FL and the instantaneous ATP concentration (see Appendix A).

The light production by immobilized FL upon addition of limiting [ATP] is also given in Figure 1. The steady-state light intensities observed at low [ATP] with the same amounts of soluble and immobilized FL should be similar since, due to the slow turnover of FL, the local concentration of ATP in the beads approximates that in the medium (see Appendix B and Figure 6a). At higher [ATP], the comparison between both systems is complicated by a larger extent of product inhibition in the case of the immobilized system, due to accumulation of products in the environment of FL. Nevertheless, control experiments have shown that the relative activities of both systems differ significantly only in the intermediate range (1–1000 μM) of ATP concentrations, while at very low or very high [ATP] they are comparable.

A striking difference between the two FL preparations is that the immobilized system responds more slowly to the addition of ATP than the soluble enzyme. This effect is due to a slower diffusion of substrate to the microenvironment of the enzyme in the first case.

The table in Figure 1 reports the kinetic parameters for the lag and the rise of the light output, calculated by fitting the data in Figure 1 to eq A2, using a curve fitting program (Bevington, 1969). The uncertainty for the real time of ATP addition (time between the injection and the actual mixing) was compensated by calculation (see legend to Figure 1). The data with soluble FL are in agreement with previously reported values calculated from stopped-flow measurements (DeLuca & McElroy, 1974; Lemasters & Hackenbrock, 1976). In the immobilized system, both the lag and rise times are retarded to the same extent compared with the soluble system. In this case, immobilized FL actually monitors the concentration of ATP diffusing from the well-mixed bulk solution into the beads.

Assuming that this equilibration of ATP takes place as a pseudo-first-order process, which is rate limiting in the reaction, an estimate for the rate of diffusion into the bead may be calculated from the linear portions of semilogarithmic plots of the data (light intensity vs. time) for the immobilized enzyme. A typical value is 1.3 s^{-1}. This estimate has no defined physical meaning, since it is a combination of kinetic constants, but it represents an easily determined parameter for comparative purposes.

A problem encountered in measurements with immobilized FL at a relatively long time range (depending on the turnover of the FL reaction) is the accumulation of product inside the FL beads when no external mixing is operating, leading to the decay of light output. On the other hand, with the enzyme in solution, a slow but significant denaturation of FL is observed upon mixing. These difficulties were circumvented by avoiding lengthy measurements with immobilized FL in unstirred media and with soluble FL in stirred solution.

Due to the intrinsic properties of FL, light emission tends to lag after the variation in [ATP] near the enzyme. However, this difficulty can be overcome if the rate constants k_1 and k_2 are known or if [ATP] variation is set experimentally at a much lower rate. Thus, under proper experimental conditions, FL represents a noninteracting probe for ATP generation or utilization in the immediate environment of the enzyme.

In order to illustrate some applications of FL as a localized probe for ATP, we studied the evolution of light output from immobilized FL during the course of ATP production by immobilized PK or its utilization by immobilized HK. In each case, we used FL immobilized on separate beads to monitor steady-state levels of ATP in the bulk medium and coimmobilized FL as a probe for ATP inside the bead.

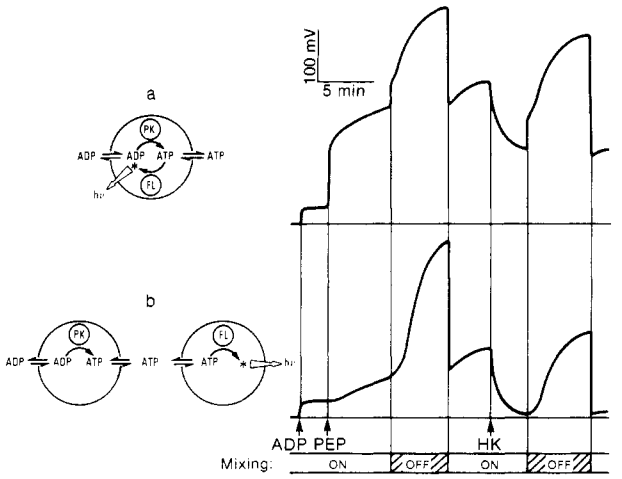

FIGURE 2: Light evolution with immobilized FL and PK. The reaction conditions were as described under Materials and Methods. The reaction mix contained 2.5 mg/mL coimmobilized FL–PK from preparation IV (a) or 2.9 mg/mL immobilized FL from preparation I and 2.3 mg/mL immobilized PK from preparation II (b) (see Table I). The final concentration for the added reactants was as follows: ADP, 1 μM; PEP, 1 mM; HK, 2 IU/mL. Glucose was at a final concentration of 10 mM. The mixing protocol is indicated by the bar at the bottom of the figure.

Studies with (Co)Immobilized FL and PK

Accumulation of ATP in the Microenvironment of Immobilized PK. Upon addition of ADP and PEP to immobilized PK, a sequence of events is initiated that includes diffusion of substrates into the beads, their transformation by the immobilized enzyme, and diffusion of products (ATP and pyruvate) to the bulk medium. The variation of light intensity vs. time for such an experiment in the presence of separately or coimmobilized FL is given in Figure 2. When ADP is added, an increase in light output is observed, due to the presence of contaminating ATP in the ADP solution (1.2%). Control experiments with both soluble and immobilized FL in a standard ATP solution showed that the light response is linear with ATP concentration over the range of luminescence used in Figure 2. On addition of a saturating amount of PEP, ATP is generated inside the beads bearing PK. This is expressed in either case as an increase in the light output from immobilized FL. With the coimmobilized system (a), in which the newly formed ATP is immediately monitored by the close-by FL, a burst is first observed followed by a linear increase. This indicates that ATP accumulates rapidly inside the beads and then it diffuses more slowly to the bulk medium. On the other hand, with separately immobilized FL and PK (b), a lag in light evolution occurs previous to the linear increase, indicating that the initial ATP production in the PK beads is not detected by FL immobilized on different beads. A similar pattern is observed with immobilized PK and soluble FL (not shown), and the slopes for the linear increase are equivalent in all cases, with the same amount of immobilized PK.

Effect of Mixing. When the mixing is stopped during ATP synthesis (Figure 2), a rapid burst in light output from the coimmobilized FL–PK beads is observed, while light evolution from separately immobilized FL remains undisturbed for the same period. We expect that stirring of the bulk medium helps to facilitate the removal of ATP from the microenvironment by efficiently reducing its concentration in the unstirred layer adjacent to the bead. Thus, the burst represents a secondary accumulation of ATP in the environment of immobilized PK, due to a new steady state for the local [ATP] between its formation and slower diffusion out into the unstirred solution. Upon further incubation, the beads begin to settle down so they eventually form a pellet. Consequently, ATP is expected to accumulate further in the unstirred layer around the beads in which it diffuses more slowly (since the sedimentation process creates turbulence by itself). Again, this results in an increase in light output. This effect is also apparent with FL and PK immobilized on separate beads because the accessibility of ATP to the FL beads is increased due to a proximity effect.

When the mixing is restored, the accumulated ATP is rapidly diluted, the beads are resuspended, and the system returns to the original steady state. The accumulation of ATP in the environment of the bead is a true kinetic effect, depending on PK activity. Indeed, in the presence of ATP with immobilized FL only, no increase in light output is observed when the mixing is stopped. This control experiment rules out a preferential partition of ATP inside the beads due to weak interactions with the support (Engasser & Horvath, 1976; Goldstein, 1976; Laidler & Bunting, 1980).

Effect of HK as a Trapping System for ATP. It can be predicted that addition of soluble HK and glucose to immobilized PK actively producing ATP will result in the reduction of [ATP] in the bulk solution.

When HK is added to separately immobilized FL and PK, the light signal decreases exponentially to a very low value (Figure 2, trace b). This value represents the new ATP concentration present in the bulk medium when a steady state is reached between its production by immobilized PK and its consumption by HK (Aflalo & Shavit, 1982). Under these conditions [ATP] is inversely proportional to the efficiency of the HK trap (Rosen et al., 1979). Thus, the great excess of HK added is able to convert most of the ATP released into the medium to ADP and glucose 6-phosphate. When HK is added to coimmobilized FL and PK, the light intensity is reduced (trace a), following the same kinetics as in the former case. However, the new steady-state value is much higher and corresponds closely to the initial burst observed with the onset of PK activity. This result strongly suggests that soluble HK has poor access to the interior of the bead, so it could not compete with coimmobilized FL for ATP generated inside the bead. The addition of a second aliquot of soluble HK does not significantly reduce the signal further, indicating saturation with HK.

When the mixing is stopped, a biphasic increase in light intensity is still observed, in spite of the presence of soluble HK, which should "pull" ATP out of the bead. However, the kinetic effect of a slower diffusion of ATP at the interface still prevails and enables its accumulation in the environment of immobilized PK. While the beads are settling down, the ratio between PK and HK activities increases in their environment, since HK remains homogeneously distributed. Consequently, the concentration of ATP around the beads gradually increases up to a new steady-state value. This value is lower than that reached with each system in the abscence of HK.

A similar experiment was done with HK immobilized on separate beads. In this case, the slow increase in light output was essentially abolished due to cosedimentation of both immobilized PK and HK. Nevertheless, the transient increase observed with the coimmobilized FL–PK system was conserved. These phenomena illustrate the relation between the concentration of ATP in the open system of the bead to that in the medium.

The results presented above demonstrate the accumulation of ATP in the microenvironment of immobilized PK. This effect can be directly quantitated with coimmobilized FL as

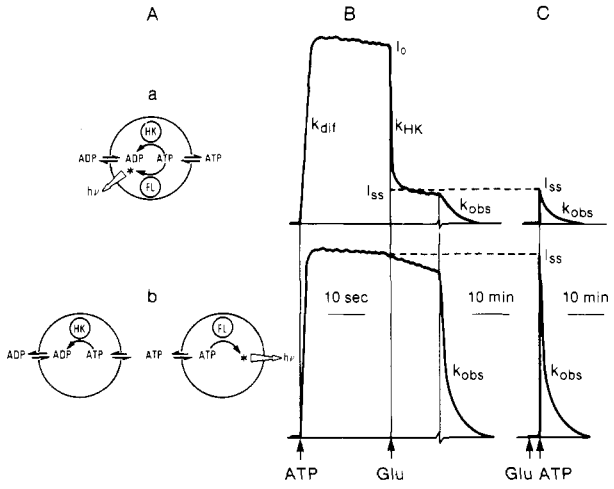

FIGURE 3: Variation of light output with immobilized FL and HK. The reaction conditions were as described in Figure 2. The reaction mix contained 5.0 mg/mL coimmobilized FL–HK from preparation V (a) or 4.6 mg/mL immobilized FL from preparation I and 4.9 mg/mL immobilized HK from preparation III (b) see Table I). The final concentration for the added reactants was as follows: ATP, 100 nM; glucose, 0.2 mM. The suspensions were stirred during the whole experiment. k_{dif}, k_{HK}, and k_{obsd} represent the pseudo-first-order rate constants for the diffusion-limited light evolution and the fast and the slow decays of light output in (b), respectively. (A) Schematic representation of the (co)immobilized systems; (B) transcient depletion of ATP in the microenvironment of HK; (C) steady-state measurements.

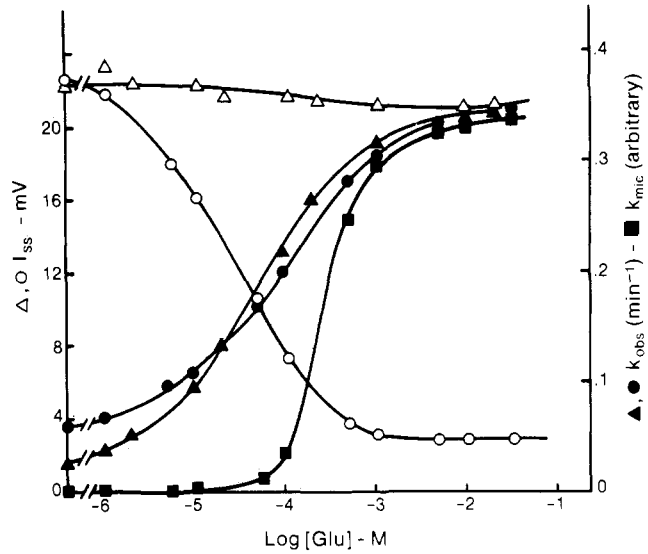

FIGURE 4: Effect of glucose concentration with immobilized FL and PK systems. The reaction conditions were as described in Figure 3C. The indicated glucose concentrations were added 1 min before the addition of ATP. The circles and triangles represent data for coimmobilized FL–HK and FL and HK immobilized on separate beads, respectively. Open symbols stand for light intensities (left) and black symbols for rate constants (right). The squares are for the microscopic rate constants for HK in the coimmobilized system, calculated with $k_{int} = k_{obsd}/\mu$, with μ representing the ratio of I_{55} in the presence of glucose to that in its absence (see text).

a localized probe but not with macroscopic measurements.

Studies with (Co)Immobilized FL and HK

Depletion of ATP in the Microenvironment of Immobilized HK. Using a similar approach, we assessed the respective activities of both enzymes separately by sequential addition of ATP and glucose. Typical measurements of the time course of light output are presented in Figure 3.

When ATP is added in the absence of glucose (Figure 3B) to coimmobilized FL–HK (a) or separately immobilized FL and HK (b), a rapid light evolution is observed followed by a very slow decay. This particular point will be discussed later. We may assume that ATP is evenly distributed in the beads and the medium and that the light intensity is proportional to [ATP] (see Appendix A).

After the rapid injection of glucose which promotes HK activity, a monophasic decay of light intensity is observed with the separately immobilized FL and HK. However, with the coimmobilized system, the light intensity decays in a biphasic pattern. The fast phase represents the approach to a new steady state for ATP in the beads between its consumption by immobilized HK and its diffusion inward from the bulk medium. Since the volume of the medium is much larger than the pore volume of the beads, this steady state is sustained until the complete depletion of ATP from the medium, which is represented by the slower decay in light output observed in both systems. Indeed the calculated rate constants for the pseudo-first-order slow decays (k_{obsd}) in mechanisms a and b of Figure 3B are very close. As expected, light intensity in the separately immobilized system reflects the variation of ATP concentration in the bulk medium only.

In spite of identical amounts of FL being present in both cases, the peak light intensity is lower in the coimmobilized system. It is well-known that in the absence of glucose HK acts as an ATPase with a catalytic ability of about $^1/_{40}$ that measured in its presence (Colowick, 1973). This activity, significant in the microenvironment of the HK bead, may be responsible for the slow decay of light intensity and the difference in light output observed in the presence of ATP only.

When glucose is added before ATP, injection of the latter results in a fast rise in light intensity followed by a slower decay with both systems (Figure 3C). However, the peak light intensity for coimmobilized FL–HK (a) is much lower than that of the separately immobilized system (b). These values reflect the respective steady states of ATP in the FL beads as described above.

The ratio between those values is very similar to that of the respective light intensities at steady state measured just before and just after addition of glucose $(I_{ss}/I_0)^2$ in Figure 3B, with the coimmobilized system. This experimentally determined parameter is extremely useful since it represents the effectiveness factor for immobilized HK and permits the calculation of intrinsic kinetic parameters of the heterogeneous system (see Appendix B).

Dependence of Measured Parameters on Immobilized HK Activity. We assume that the local activity of immobilized HK has the same dependence on glucose concentration as the enzyme in solution. Thus, we may control experimentally HK activity by varying glucose concentration. The experiments described in Figure 3C were repeated at different glucose concentrations. Glucose was added in excess over ATP and was allowed to diffuse inside the bead before the addition of the latter. Under these conditions, glucose concentration inside the beads should remain constant during catalysis. The data for coimmobilized FL–HK and the separately immobilized reconstituted system are given in Figure 4. Increasing glucose concentration in both systems results in a gradual increase of the macroscopic rate constant for ATP consumption (k_{obsd}) up to a saturation value. We expect that the dependence of this parameter on glucose concentration will also reflect the diffusional restriction imposed on ATP inside the beads. The

[2] The notation used for light output is I_0 for soluble FL and I_{ss} for immobilized FL coupled to other processes (diffusion and/or catalysis). With immobilized FL at steady state in the absence of coimmobilized enzyme activity, $I_{ss} = I_0$.

higher the ratio between the local activity of HK and the diffusion coefficient of ATP (α_{HK}, see eq A7), the more severely will the macroscopic reaction be limited by diffusion. Diffusion control may be quantitated by an effectiveness factor (μ_{HK}, see eq A10), defined as the ratio of the velocity of the immobilized HK to that of the same amount of enzyme in solution.

The initial light intensity observed with FL and HK immobilized on separate beads is not significantly affected by glucose. However, with coimmobilized FL–HK, this parameter is reduced to a finite value when glucose concentration is increased. Neglecting the slow ATPase activity of HK in the absence of glucose, the effectiveness factor for the coimmobilized system may be calculated as the ratio of the peak light intensity at each glucose concentration to that measured in its absence (eq A13). This value permits in turn the estimation of the microscopic rate constant (k_{mic}) for ATP consumption inside the bead with

$$k_{mic} = (V_m/V_b)k_{obsd}/\mu_{HK}$$

where V_m and V_b represent the volume of the medium and the pore volume of the beads, respectively. Normalized values for calculated k_{mic} are included in Figure 4. A comparison of the dependence of microscopic and macroscopic activity on substrate concentration stresses the effect of diffusion in such heterogeneous systems. As the microscopic activity of HK increases, α increases and μ decreases (see Appendix B and Figure 6), so that the kinetic behavior of the heterogeneous system departs more from that in the ideal case of an enzyme in a well-stirred solution (Katchalski et al., 1971; Engasser & Horvath, 1976; Goldstein, 1976). At saturating glucose concentration, the effectiveness of immobilized HK in preparation V to consume low ATP concentrations is about 0.11. By use of an approximation for the pore volume of the beads (0.5 mL/g), an intrinsic rate constant for the local activity of HK of 1270 min^{-1} may be calculated.[3] If the average radius of the beads is taken as 50 μm, an effective diffusion coefficient for ATP in the beads of 0.5×10^{-4} cm^2/min is derived from the equations in Appendix B. This value is lower than that expected for ATP in water (about 3×10^{-4}) and is reasonably comparable with those reported in the literature for small molecules in similar systems (Katchalski et al., 1971; Engasser & Horvath, 1976; Goldstein, 1976; Laidler & Bunting, 1980).

Dependence of Measured Parameters on Beads Concentration. The kinetic behavior of a heterogeneous enzymatic system is represented by two sets of parameters: microscopic values, inherent to the system, and macroscopic parameters, determined empirically, which describe its effective properties. The former are expected to be independent of the total amount of immobilized enzymes since they define intrinsic physicochemical properties of the system. We studied the dependence of several experimentally derived parameters on the concentration of beads, using coimmobilized FL–HK.

The light intensity at steady state in the presence of ATP alone (I_0) or its value in the presence of glucose (I_{ss}), extrapolated as in Figure 3Ba to the time of glucose addition) is directly proportional to the concentration of the beads (Figure 5, top). As shown in Appendix B (eq A13 and A14), these values are functions of the total amount of FL, which

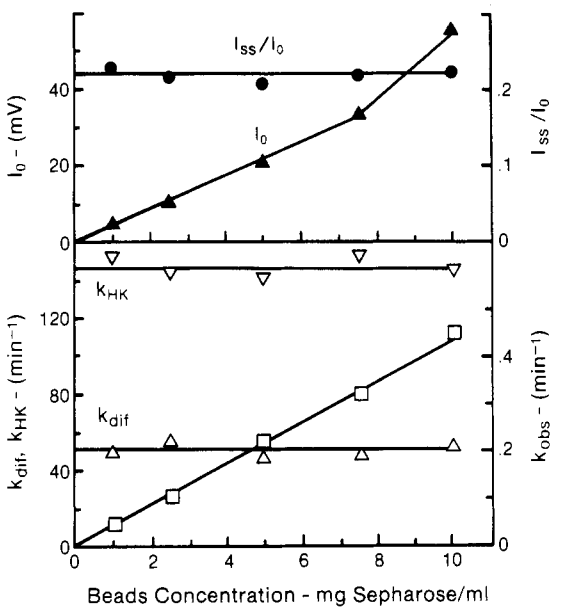

FIGURE 5: Effect of bead concentration on estimated kinetic parameters for coimmobilized FL–HK. The reaction conditions for coimmobilized FL–PK from preparation V and the measured parameters were as described in Figure 3Ba, except that the concentration of the beads was varied as indicated.

depends on the amount of beads in suspension. At higher concentrations of beads, an upward curvature is observed, which may indicate a decrease in product inhibition due to a higher FL:ATP ratio in these conditions. Nevertheless, the relation between I_{ss} and I_0 remains constant at any concentration of beads. As mentioned in the above section, the ratio I_{ss}/I_0 represents the effectiveness factor for HK, which is a function of intrinsic properties of the system only.

The rate constants for the pseudo-first-order processes occurring rapidly upon addition of ATP (k_{dif}) and glucose (k_{HK}) were derived from the linear portion of semilogarithmic plots of data similar to those described in Figure 3Ba. The rate constant of the subsequent slow process (k_{obsd}) was determined similarly, and the results are presented in Figure 5 (bottom). The latter parameter is a function of the total amount of HK and thus proportional to the concentration of the beads. However, k_{dif} and k_{HK} (which depend on the rate of diffusion and the local activity of HK) represent intrinsic properties of the system and are independent of the concentration of beads.

Conclusions and Perspectives

The approach described above permits a direct evaluation of intrinsic parameters of enzymes sequestred at high concentrations in a definite compartment simulating in vivo conditions. The physicochemical properties of the microenvironment (buffer capacity, fixed charges density, dielectric properties, viscosity, etc.) may be varied during the immobilization procedure. However, the various calculations are based on assumptions that may not hold for artificial immobilized systems (no effect of immobilization on the intrinsic parameters of the enzymes, homogeneity of the beads, etc.). We expect for instance that the enzymatic activity on small beads will be much less affected by diffusion and thus contribute more to the total observed activity than the activity on larger beads. Additional investigation is needed to relate quantitatively the experimental data with theoretical considerations. At this stage, the analytic approach used above is valid for very low substrate concentrations only, where the catalytic velocity is pseudo-first-order and product inhibition of FL is negligible. The generalization of local [ATP] mon-

[3] Assuming a K_m of 100 μM for ATP, the specific activity of immobilized HK is 63.6 IU/g (compare with 11.5 IU/g in Table I, from spectrophotometric measurements). This discrepancy may be attributed to the inadequacy of spectrophotometric measurements, made at relatively nonsaturating substrate concentrations with the immobilized HK preparations.

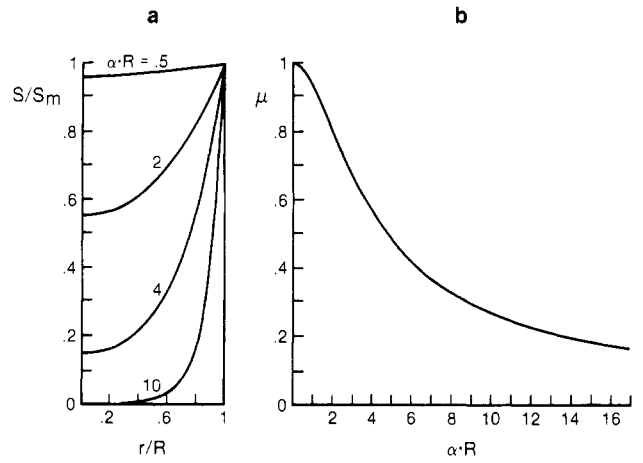

FIGURE 6: Diffusion control in a spherical heterogeneous catalytic system: (a) concentration profile of substrate in the support at steady state; (b) variation of the effectiveness factor with the physicochemical properties of the system. The curves were generated by computer according to eq A7 and A9, respectively.

itoring in more complicated cases requires the resolution of the phenomenological differential equations of the system (e.g., eq A5) by computer-assisted numerical techniques, which also permit an evaluation of the time dependence of the local substrate concentration [see Kernevez (1980)]. Nevertheless, the use of FL enables the kinetic resolution of the variation of ATP in the microenvironment of the enzyme from that in the bulk phase. This tool may add a new dimension to studies on the kinetic behavior of sequestered or surface-bound enzymes, generally assessed by indirect macroscopic measurements in both artificial and isolated biological systems [for example, see Rosen et al. (1979) and Aflalo and Shavit (1982)].

The concept of a localized probe for ATP can also be extended to cellular systems. The firefly luciferase gene has been successfully cloned and expressed in bacteria, plants, and cultured animal cells (deWet et al., 1985, 1987; Ow et al., 1986). However, the analysis of light output in vivo is complicated by a low permeability of luciferin through membranes and the uncertainty of the intracellular location of luciferase. It is now possible to target the enzyme into various cellular compartments by use of the products of fusion between the FL gene and that of various leader sequences [for review, see Colman and Robinson (1986)]. With such a biologically directed probe, a new survey of the intermediary metabolism of ATP may be initiated. Such studies might provide insights on the transfer of ATP between cellular compartments and the energy coupling between metabolic processes.

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APPENDIX

(A) Kinetic Analysis of the Firefly Luciferase Reaction

In a homogeneous solution, when a low concentration of ATP is added to FL in the presence of saturating LH_2, a steady state in light production $(I_0)^2$ is rapidly established at which

$$I_0 = c(\mathrm{d}h\nu/\mathrm{d}t) = c(\mathrm{vol})k_{FL}[FL][ATP] \qquad (A1)$$

where c is a constant depending on the geometry and physical properties of the luminometer used, vol is the reaction volume, and k_{FL} is the pseudo-second-order limiting rate constant for the reaction in these conditions.

Pre-Steady-State Kinetics at Limited [ATP]. Upon addition of ATP, light production might be represented by a combination of two pseudo-first-order processes occurring in sequence [see Adamson (1973)]. The system reaches eventually a steady state at which a constant light output is observed. The time dependence of luminescence (proportional to [FL·P*], see Results) is then

$$I = I_0\left[1 - e^{-k_1 t} - \frac{k_1}{k_1 - k_2}(e^{-k_2 t} - e^{-k_1 t})\right] \qquad (A2)$$

where k_1 and k_2 are the pseudo-first-order rate constants for the lag and the rise of luminescence, respectively.

(B) Kinetic Behavior of Immobilized Enzymes

Effect of External Diffusion. Due to the action of an enzyme immobilized on a solid support, the local concentration of substate (S) should be lower than that in the medium (S_m). At steady state, the rate of substrate disappearance from the medium (Fick's first law) is equal to that of catalysis at the interface (Katchalski et al., 1971; Engasser & Horvath, 1976; Goldstein, 1976). Thus

$$-\frac{\mathrm{d}S_m}{\mathrm{d}t} = D_m A\frac{\mathrm{d}S}{\mathrm{d}x} = \frac{D_m A}{\delta}(S_m - S) = h(S_m - S) = \frac{V_{max}S}{K_m + S} \qquad (A3)$$

where D_m, A, δ, and h represent the diffusion coefficient of substrate in bulk medium, the area of the support exposed to the medium, the width of a hypothetical unstirred layer around the support, and a mass transfer coefficient. At low S ($S \ll K_m$), we get

$$S = \frac{S_m h}{V_{max}/K_m + h} \qquad (A4)$$

The extent of external diffusion control may be substancially reduced by efficiently mixing the bulk solution (apparent increase of D_m or decrease of δ in eq A3).

Effect of Internal Diffusion. When an enzyme is embeded in a porous matrix, substrate is subject to two simultaneous processes: diffusion in the matrix and its utilization by the enzyme. Introducing Fick's second law, the phenomenological equation for the system at any point of a spherical matrix of radius R is (Regan et al., 1974)

$$\frac{\mathrm{d}S}{\mathrm{d}t} = \frac{D}{r}\frac{\mathrm{d}^2(Sr)}{\mathrm{d}r^2} - \frac{V_{max}S}{K_m + S} \qquad (A5)$$

at any $t > 0$ and $0 < r < R$, where D represents the effective diffusion coefficient of substrate in the support. At steady state and for very low S, we get after rearrangement

$$\frac{\mathrm{d}^2(Sr)}{\mathrm{d}r^2} = \frac{V_{max}}{K_m D}(Sr) = \frac{k_{cat}[E]}{K_m D}(Sr)$$

$$\text{boundary conditions: } S = S_m \text{ at } r = R \qquad (A6)$$

$$\mathrm{d}S/\mathrm{d}r = 0 \text{ at } r = 0$$

The boundary conditions were set assuming that steady state is reached rapidly (no significant variation in S_m), that external diffusion is negligible, and that the enzyme concentration [E] is constant throughout the whole support.

The analytic solution is (see Figure 6a)

$$S = S_m\frac{R}{\sinh(\alpha R)}\frac{\sinh(\alpha r)}{r} \qquad \text{at } 0 < r \le R$$

$$S_0 = S_m\alpha R/\sinh(\alpha R) \qquad \text{at } r = 0 \qquad (A7)$$

where

$$\alpha = \left(\frac{k_{cat}[E]}{K_m D}\right)^{1/2}$$

The disappearance of substrate from the medium (at the interface) represents the macroscopic activity. For a single bead, applying Fick's first law, it is given by

$$-\frac{dS_m}{dt} = \frac{D}{V_m} 4\pi R^2 \left(\frac{dS}{dr}\right)_R = 4\pi \frac{RD}{V_m}[\alpha R \coth(\alpha R) - 1] S_m \quad (A8)$$

where V_m represents the volume of bulk medium. For the same amount of enzyme in solution, the velocity would be

$$v = \frac{k_{cat}[E]}{K_m} \frac{4\pi R^3}{3V_m} S_m \quad (A9)$$

We may define μ, an effectiveness factor, as the ratio between both quantities. Division of eq A8 by eq A9 gives after rearrangement (see Figure 6b)

$$\mu = \frac{3}{\alpha^2 R^2}[\alpha R \coth(\alpha R) - 1] \quad (A10)$$

This quantity is independent of the number of beads in suspension.

Quantitation of Light Output from (Co)Immobilized FL. At low [ATP], the measured light output is still given by eq A1 but in its differential form, integrated over the volume of the spherical bead, since [ATP] may vary within the support (see above):

$$I = ck_{FL}[FL] \int_0^R [ATP]_r 4\pi r^2 \, dr \quad (A11)$$

where [FL] represents the concentration of FL in the support. At steady state, we may substitute $[ATP]_r$ using eq A7. After integration and rearrangement, we get for light output at steady state $(I_{ss})^2$

$$I_{ss} = c\frac{4}{3}\pi R^3 k_{FL}[FL][ATP]_m \frac{3}{\alpha^2 R^2}[\alpha R \coth(\alpha R) - 1] \quad (A12)$$

Thus, for FL coimmobilized with another ATP consuming enzyme, eq A12 reduces to

$$I_{ss} = I_0 \mu \quad (A13)$$

where I_0^2 represents the ligth output obtained at steady state with the same amount of soluble FL (cf. eq A1) and μ stands for the effectiveness factor of the coimmobilized enzyme.

However, for singly immobilized FL, assuming a negligible rate for ATP consumption ($\alpha_{FL} \rightarrow 0$ and $\mu_{FL} \rightarrow 1$, see Figure 6), we get

$$[ATP]_r = [ATP]_m \text{ and } I_{ss} = I_0 \quad (A14)$$

References

Adamson, A. W. (1973) in *Textbook of Physical Chemistry*, pp 721–724, Academic, New York.

Aflalo, C., & Shavit, N. (1982) *Eur. J. Biochem. 126*, 61.

Axen, R., Porath, J., & Ernbock, S. (1967) *Nature (London) 214*, 1302.

Bevington, P. R. (1969) in *Data Reduction and Error Analysis for Physical Sciences*, pp 204–246, McGraw-Hill, New York.

Bowie, L. J. (1978) *Methods Enzymol. 57*, 15.

Colman, A., & Robinson, C. (1986) *Cell (Cambridge, Mass.) 46*, 321.

Colowick, S. P. (1973) *Enzymes (3rd Ed.) 9*, 1–48.

DeLuca, M. (1984) *Curr. Top. Cell. Regul. 24*, 189.

DeLuca, M., & McElroy, W. D. (1974) *Biochemistry 13*, 921.

DeLuca, M., & McElroy, W. D. (1978) *Methods Enzymol. 57*, 3.

De Pierre, J. W., & Ernster, L. (1977) *Annu. Rev. Biochem. 46*, 201.

deWet, J. R., Wood, K. V., Helinski, D. R., & DeLuca, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7870.

deWet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., & Subramani, S. (1987) *Mol. Cell. Biol. 7*, 725.

Engasser, J.-M., & Horvath, C. (1976) *Appl. Biochem. Bioeng. 1*, 127.

Goldstein, L. (1976) *Methods Enzymol. 44*, 397.

Katchalski, E., Silman, I., & Goldman, R. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol. 34*, 445.

Kernevez, J.-P. (1980) in *Studies in Mathematics and Its Applications* (Lions, J.-L., Papanicolaou, G., & Rockafellar, R. T., Eds.) Vol. 10, North-Holland, Amsterdam.

Laidler, K. J., & Bunting, P. S. (1980) *Methods Enzymol. 64*, 227.

Lemasters, J. J., & Hackenbrock, C. R. (1976) *Eur. J. Biochem. 67*, 1.

Mosbach, K. (1978) *Curr. Top. Cell. Regul. 14*, 197.

Neet, K. E., & Ainslie, G. Rr., Jr. (1980) *Methods Enzymol. 64*, 192.

Ottaway, J. H., & Mowbray, J. (1977) *Curr. Top. Cell. Regul. 12*, 107.

Ow, D. W., Wood, K. V., DeLuca, M., deWet, J. R., Helinski, D. R., & Howell, H. H. (1986) *Science (Washington, D.C.) 234*, 856.

Regan, D. L., Lilly, M. D., & Dunnill, P. (1974) *Biotechnol. Bioeng. 16*, 1081.

Rosen, G., Gresser, M., Vinkler, C., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 10654.

Schram, E., Ahmad, M., & Moreels, E. (1981) in *Bioluminescence and Chemiluminescence* (DeLuca, M., & McElroy, W. D., Eds.) pp 491–496, Academic, New York.

Srere, P. A. (1984) *Trends Biochem. Sci. (Pers. Ed.) 9*, 382.

Weber, J. P., & Bernhard, S. A. (1982) *Biochemistry 21*, 4189.

Wiehnhausen, G., Kricka, L. J., Hinkley, J. E., & DeLuca, M. (1982) *Appl. Biochem. Biotechnol. 7*, 463.

Worthington Manual (1978) *Enzymes and Related Biochemicals*, pp 98, 178, Millipore Corp., Bedford, MA.